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Potential Pathogens in the Environment: *Klebsiella pneumoniae*, a Taxonomic and Ecological Enigma[1]

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A nitrogen-deficient medium and m-Endo agar were employed in the isolation of members of the tribe *Klebsielleae* from surfaces of vegetables and seeds. With m-Endo agar at an incubation temperature of 37 C, nearly 50% of the vegetables and seven out of seven seed samples yielded organisms which biochemically and serologically were identified as *Klebsiella pneumoniae*, Viable counts were generally in the range of 10^3 cells per g of vegetable peel or seed. Organisms classified as *K. pneumoniae* exhibited seven different IMViC patterns, with the −−++, +++, and −+++ patterns most common. Seven of the eleven *K. pneumoniae* serotypes encountered have previously been isolated from human urinary tract and other infections. Fifty percent of the 40 *K. pneumoniae* examined exhibited positive acetylene-reducing activity, i.e., they possessed the capability for fixing N_2. Vegetables containing *K. pneumoniae* may constitute a potential reservoir for human nosocomial genitourinary or other infections.

The tribe *Klebsielleae* contains three genera of lactose-fermenting bacteria, *Klebsiella*, *Enterobacter*, and *Serratia* (3, 11, 15). *Klebsiella pneumoniae* is commonly associated with human respiratory and genitourinary infections. Although *Enterobacter aerogenes* (formerly *Aerobacter*) has been implicated in urinary tract infections (12, 30), it is more commonly regarded as an inhabitant of soil, plants, and the aquatic environment (3, 4, 6, 9). *Enterobacter cloacae* frequently found in sewage, water, and soil (4) has also been isolated from human bacteremias, urinary tract and other infections primarily nosocomial in nature (5, 30).

There are numerous reports in the older literature describing an association of *Aerobacter* with dairy products, plants, soil, pulp mill effluents, etc. (4, 10, 27). Since motility and decarboxylase enzymes were not studied, it is not possible to determine whether these environmental isolates were *K. pneumoniae* or *E. aerogenes*. The isolation of saprophytic bacteria from the environment, which biochemically and serologically are classified as *K. pneumoniae*, could have important microbiological consequences. As a result of the ubiquitous occurrence of organisms with the −−++ IMViC reaction in nature (17, 18, 27), the recent reports on the association of *K. pneumoniae* with sugar cane and cane juices (25), and the apparent *K. pneumoniae* colonization of guinea pigs fed on a diet of sweet potatoes (2), we have undertaken a survey to determine the incidence of *K. pneumoniae* from other common environmental sources. In this study we assess several procedures for isolating and enumerating *K. pneumoniae* from vegetables and seeds, and compare the biochemical properties of environmental isolates with human strains of *K. pneumoniae*.

MATERIALS AND METHODS

Organisms. *K. pneumoniae* M5a-1 and UW1 were isolated from a 2,3-butanediol fermentation and milk, respectively, and were supplied by P. W. Wilson (23). Soil isolates Ka-1 and Ka-2 were obtained from M. A. Line and M. W. Loutit (22). Human clinical isolates of *K. pneumoniae* were supplied by the University of Oregon Medical School (UOMS 1-6) and the Medical University of South Carolina (MUSC 1, 2, 4, and 5). Six *K. pneumoniae* cultures (R301-R306) were isolated from Oregon rivers in the vicinity of paper and pulp mill effluents using conventional Millipore filter techniques and fecal coliform selective medium (Bacto m-Endo agar incubated at 37 C; 14). The *K. pneumoniae* type culture (ATCC 13883) was obtained from the American Type Culture Collection, Bethesda, Md. Nine cultures were obtained from living white fir trees by using our previously described procedures (29). The remaining organisms designated by the prefix "V" were isolated from vegetables (V54-V145 and V177-V244) or vegetable seeds (V150-V176) obtained at retail markets or from vegetables in a home garden.

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Isolation procedures. Early isolation attempts utilized a modified Hino and Wilson medium (19, 29). Selection of this medium was made on the basis of the known ability of many *K. pneumoniae* to fix atmospheric N_2 (23). Vegetables were aseptically peeled into tared sterile beakers, a measured volume of sterile 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) was added and the contents were shaken for 5 min at 22 C. Serial dilutions were plated on N_2-deficient medium using the spread-plate technique. The plates were placed in a jar which was then filled with a mixture of 80% nitrogen and 20% argon and was maintained under a slight positive pressure. After incubation at 30 C for 3 to 5 days, the large, mucoid, translucent colonies were enumerated. Typical colonies were purified by transferring them several times on N_2-deficient medium and then to nutrient agar. An attempt was made to eliminate many of the common *E. aerogenes* from the N_2-deficient plates by employing a higher incubation temperature (37 C) in some trials.

Other isolation trials involved the use of MacConkey agar (Difco), EMB, and m-Endo agar (Difco) incubated at 37 C. In our hands, MacConkey agar proved unsatisfactory due to variable colony coloration by different isolates of known *K. pneumoniae*. m-Endo agar was preferred over EMB since the green metallic sheen of the *Klebsiella* colonies was more readily observable. Vegetables were treated in the same way as with the N_2-fixing method. Raised, mucoid colonies with a green sheen on m-Endo agar were isolated. In one trial, samples were incubated at both 42 and 37 C. Five typical colonies were picked from each dilution per vegetable on the 42 C plates and usually two to three colonies were picked from the 37 C plates. The following vegetables yielded *K. pneumoniae*: white potato, red potato, mushroom, radish, radish tops, carrots, sweet potato, cucumber, and green onion. No *K. pneumoniae* isolates were obtained from celery, beets, lettuce, or broccoli.

In other experiments seeds were emptied into sterile beakers, covered with sterile 0.01 M Tris buffer (pH 7.5), and shaken. After 30 min, dilutions were plated onto m-Endo agar and incubated at 37 C. The beakers with seeds were also incubated statically at 37 C for 24 h. Additional Tris buffer was added to keep the seeds moist, and dilutions were again prepared for plating onto the selective medium. *K. pneumoniae* were isolated from all the seeds examined. These included radish, carrot, rutabaga, turnip, lettuce, corn, and cucumber.

Biochemical studies. Testing methods adhered to those recommended in the *Manual of Clinical Microbiology*, and all media were from Difco Laboratories (3). Cultures were incubated at 37 C. The acetylene-reduction method was employed as biochemical evidence of N_2 fixation. This procedure relies on the substitution of acetylene for N_2 gas as the substrate for nitrogenase. The ethylene formed is detected and quantitated in a gas chromatograph (7).

RESULTS

In an attempt to specifically isolate *K. pneumoniae* from environmental samples, two selective media were employed using three incubation temperatures. Table 1 summarizes these results. The nitrogen-deficient Hino and Wilson medium proved to be the least successful of the procedures tried, and *K. pneumoniae* were only isolated when the medium was incubated at 37 C. On the other hand, m-Endo agar proved to be a dependable selective medium for detecting *K. pneumoniae* in the samples studied. On this medium, presumptive *K. pneumoniae* were chosen on the basis of rapid growth and colony morphology.

Estimated numbers of *K. pneumoniae* were in the range of 10^3/g of vegetable peel for all the trials yielding *K. pneumoniae*. Incubation for the longer period (24 h) in the second seed trial (trial 7B) allowed the rare *K. pneumoniae* to multiply and become detectable. The advantages of the selective conditions become obvious when one considers that on N_2-deficient or m-Endo agar incubated at 25 C the total counts are 10^3 to 10^4 times greater than when incubated at 37 C. At 37 C and with m-Endo agar, about one-third of the green sheen colonies appearing on the 10^{-2} dilution plates were identified as *K. pneumoniae*. The remaining green sheen colonies tested proved to be *Enterobacter* species.

The environmental and human *K. pneumoniae* isolates were subjected to 23 biochemical tests (Table 2). Characteristics common to these isolates include lysine decarboxylase activity, lack of motility, acid slant, and butt on triple-sugar-iron (TSI) agar with production of gas, no liquefaction of gelatin, and rapid fermentation of dextrose, glycerol, maltose, and mannitol. Group I isolates were uniformly positive as to urease activity, utilization of citrate, and malonate, and fermentation of lactose. Adonitol and dulcitol were also fermented in most cases. Isolates comprising group II were urease negative and variable in their utilization of citrate. Approximately 90% of the group 2 isolates utilized malonate and fermented lactose and inositol. All isolates fermented adonitol.

Fourteen of fifteen seed isolates and six of twenty-five vegetable isolates exhibited positive acetylene-reducing capacities under anaerobic conditions. Rates of acetylene reduction ranged from 2,863 to 8,237 nmol/10 ml of culture over a 24-h period.

Some investigators have correlated *Klebsiella* species differentiation with IMViC reactions (1, 8). About 50% of the environmental and human *K. pneumoniae* exhibited the $--++$ IMViC pattern, whereas 28% produced indol. In addition, 36% of the isolates were methyl-red positive. The ATCC type culture of *K. pneumoniae*

TABLE 1. *Influence of incubation temperature and selective medium on isolation of K. pneumoniae from vegetables and seeds*

Trial no.	No. samples tested	Selective medium	Incubation temp (C)	No. of + samples[a]	Numbers/g[b]
1	11 (V)[c]	Mod. H & W[d]	30	0	0
2	15 (V)	Mod. H & W[d]	30	0	0
3	19 (V)	Mod. H & W[d]	37	1	5×10^3
4	10 (V)	Mod. H & W[d]	37	3	10^3 to 2×10^3
5	7 (V)	m-Endo	37	5	3×10^3 to 2×10^5
6A	14 (V)	m-Endo	42	5	10^3 to 5×10^5
6B	8 (V)	m-Endo	37	5	2×10^3 to 3×10^5
7A	7 (S)[e]	m-Endo	37	1	<10
7B	7 (S)	m-Endo	37	7	10^3 to 2×10^3

[a] +, Samples yielding *K. pneumoniae*.
[b] *K. pneumoniae* were isolated from 10^{-2} to 10^{-5} dilution plates.
[c] Isolates obtained from vegetable peelings.
[d] Modified Hino & Wilson medium with 1% Noble agar.
[e] Isolates obtained from commercial seeds. Trials 7A and 7B include the same seeds, but in trial 7A seeds were incubated for 30 min in 0.01 M Tris (pH 7.5) at room temperature, whereas in trial 7B 0.01 M Tris and seeds were incubated at 37 C for 24 h.

gave an uncommon IMViC reaction of $-+-+$. This pattern was also reported for this strain by Cowan et al. (8). Organisms we have classified as *K. pneumoniae* exhibit a total of seven types of IMViC patterns.

Eight vegetable, eight wood, and four river isolates comprising five IMViC groups were sent to the Center for Disease Control, Atlanta, Ga., for serological typing and for confirmation of our identification. Table 3 lists the isolates examined, IMViC reactions, sources of isolation, and serological type. In all cases our identification was confirmed. Unfortunately, six of the eight wood isolates did not have sufficient capsule for typing, but eleven different serotypes were obtained among the remaining 14 cultures.

DISCUSSION

Recent literature described *K. pneumoniae* in association with sugar cane plants, juices, associated insects (25); small green pet turtles (R. H. McCoy, and R. J. Seidler, 1973. Appl. Microbiol., in press); guinea pigs, and humans fed a sweet potato diet (2); and New Zealand Tussock grassland soils (22). As a consequence of the apparent incidence of these organisms in nature, it seemed pertinent to devise a method for their isolation and enumeration. The present study confirms that *K. pneumoniae* may also be isolated from the surfaces of vegetables and even from within living trees, although the predominant organism in these habitats is *Enterobacter*.

Isolation attempts employing a modified Hino and Wilson nitrogen-deficient medium (19) proved largely unsuccessful in our hands although previous investigators indicated isolation of *K. pneumoniae* from fecal material by a similar method (13). A selective medium, m-Endo, was subsequently used. In this manner, some 30 to 50% of typical colonies on m-Endo proved to be *K. pneumoniae*, indicating that although these organisms may be outnumbered in nature, appropriate selective conditions allow for their detection and isolation.

A most disturbing difference is found in the outcome of the IMViC reactions of *K. pneumoniae* as reported by various investigators. Cowan et al. (8) as well as Bascomb et al. (1) report a $-+-+$ IMViC pattern for *K. pneumoniae*, although American diagnostic schema list a $--++$ reaction (3, 11, 15). The type culture of *K. pneumoniae* (ATCC 13883) used in the present studies and by Cowan et al. (8) has the $-+-+$ pattern. However, we found in our studies that this pattern was infrequently encountered.

Previously, there was much confusion concerning the taxonomic relationships of microorganisms belonging to the tribe *Klebsielleae*. It would appear that numerous synonyms have been used for the organism *K. pneumoniae*, including non-motile *Aerobacter aerogenes* (4, 10, 26), *Klebsiella aerogenes* (1), *K. edwardsii* (8), and *K. oxytocum* (8). Edwards and Ewing (11) would group the above cultures into the species *K. pneumoniae*. Cowan et al. (8) proposed *K. aerogenes*, *K. pneumoniae* and two varieties of *K. edwardsii*, whereas Bascomb et al. (1) consider *K. aerogenes-oxytoca-edwardsii* to constitute only one taxon separable from *K. pneumoniae*. It is not our intention to debate

which classification scheme is preferable but merely to summarize this information to allow further perspective on some of our identifications.

In view of the present and other studies which reveal *K. pneumoniae* in the environment, perhaps a re-evaluation of the sanitary water analysis may be in order. The question remains as to what proportion of the −−++, −+−+, and ++++ IMViC types are not *E. aerogenes* but, in reality, are *K. pneumoniae*, an organism ordinarily associated with human respiratory, genitourinary and intestinal habitats.

The clinical significance of *K. pneumoniae* was classically limited almost entirely to their association with lobar pneumonia (6, 9). More recently, it has been noted that these organisms are capable of causing a greater variety of infections (12, 20, 24) including institutional outbreaks of infant diarrhea (21) and suppurative conditions (6). In a recent study of 306 hospital strains, nearly 70% of the *K. pneumoniae* were implicated in urinary tract infections and 17% were obtained from various abscesses and wound infections (30). Of the environmental cultures randomly selected for serotyping, 7 of the 11 types found (7, 11, 15, 21, 23, 60 and 64) have also been isolated from human urinary tract and other miscellaneous infections (12, 30). The high numbers of *K. pneumoniae* on fresh vegetables is probably a result of contamination occurring during cultivation or preparation for marketing (16). Therefore, fresh vegetables could be a focal point for *K. pneumoniae* nosocomial infections.

As a result of the multiple drug-resistance patterns commonly encountered among human *K. pneumoniae* (12, 20, 28, 31), correct identification and selection of antimicrobial agents for treatment have become more critical. Although our isolates show no indication of multiple drug resistance to the more commonly used drugs, these cultures might possess the same potential to acquire resistance as do common clinical cultures (20). Recent studies in our department have shown this to be the case (L. R. Brown, personal communication).

TABLE 2. *Identification and biochemical properties of human and environmental K. pneumoniae*

Characteristics	Strains positive (%)[a]	
	Group I	Group II
TSI:		
Slant	100 (A)[d]	100 (A)
Butt	100 (A)	100 (A)
Gas	100	100
H_2S (7 days)	14	0
Arginine dihydrolase[b]	0	0
Lysine decarboxylase	100	100
Ornithine decarboxylase	0	0
Motility[c]	0	0
Urease (4 days)	100	0
Indole	30	11
Methyl red	20	89
Voges-Proskauer	97	67
Citrate	100	33
Malonate	100	89
Gelatin	0	0
Adonitol	74	100
Dextrose	100	100
Dulcitol	59	56
Glycerol	100	100
Inositol	93	89
Lactose	100	89
Maltose	100	100
Mannitol	100	100
N_2 Fixation	51	40

[a] Group I isolates included 44 vegetable, 9 wood, 6 river, 2 soil, 2 milk, 1 ATCC, and 9 human samples. Group II isolates included 8 vegetable and 1 human samples.

[b] Moellers decarboxylase medium (Difco).

[c] Cultures inoculated into nutrient agar deeps (0.5% agar, Difco) and examined after 2 and 7 days. Six- to twelve-h nutrient broth cultures were also examined under a microscope.

[d] A indicates acid production.

TABLE 3. *Source and serological type of some environmental K. pneumoniae*

Isolate	Source	IMViC reaction	Serological type
R 301	River	−−++	64
R 302	River	−−++	15
R 304	River	−−++	47
R 305	River	−−++	60
V 104a	Mushroom	−+++	related to 6 & 7
V 233	Radish tops	−+−+	11
V 244	Green onion	−+−+	47
V 236	Radish	−+++	21
V 112	Potato	++++	26
V 86	Potato	++++	72
V 171	Turnip seeds	−−++	23
V 151	Carrot seeds	−−++	23
27B	White fir[a]	++++	68
36C	White fir	++++	68

[a] The white fir isolates were among several hundred cultures characterized from decay in living trees. A preliminary report of this study has been published (30).

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